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Seroepidemiological Study of Human Herpesvirus-6 and -7 in Children of Different Ages and Detection of These Two Viruses in Throat Swabs by Polymerase Chain Reaction

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The presence of human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) in throat swabs of 62 children of different age groups (group I, ages 0–5 month; group II, ages 6–11 months, group III, ages 12–23 months, group IV, age 2–8 years) and 28 adults was detected by polymerase chain reaction. The detection rate of HHV-6 DNA was the highest (87%) in children aged 1-year-old and decreased with age, whereas the detection rate of HHV-7 increased with age and reached a maximum in adults. HHV-6B was detected in almost all samples except for two children who secreted only HHV-6A. When the antibody prevalence was determined in the four groups of children, HHV-6 antibody was detected in 8/12 (66.7%), 10/12 (83.3%), 15/16 (93.8%), and 13/14 (92.9%), respectively. Antibody to HHV-7 in these groups was detected in 6/12 (50.0%), 4/12 (33.3%), 12/16 (75.0%), and 13/14 (92.9%), respectively. Detection of HHV-6 DNA in throat swabs of triplets who had the sequential onset of exanthem subitum was attempted by using samples sequentially collected from these children after the onset of the disease in the first patient. HHV-6 DNA with high copy numbers was detectable during the acute and convalescent phases of the disease in all patients, but no DNA was detected in samples collected before the onset of disease.

KEY WORDS: HHV-6, HHV-7, PCR, seroepidemiology, transmission

INTRODUCTION

Human herpesvirus 6 (HHV-6) was first isolated from patients with lymphoproliferative disorders [Salahuddin et al., 1986], and it is now divided into two distinct classes designated HHV-6A and HHV-6B or variant A and B [Ablashi et al., 1993]. In 1988, we reported that HHV-6 is the causative agent of exanthem subitum (ES) [Yamanishi et al., 1988]. ES is mainly caused by HHV-6B [Dewhurst et al., 1993; Yamamoto et al., 1994]; however, the clinical features of HHV-6A have not been identified. Furthermore, tests are not yet available to distinguish HHV-6A and -6B infection by serological examination, and there are no data concerning seropidemiological study of their variants. Frenkel et al. [1990] isolated another lymphotropic herpesvirus named human herpesvirus 7 (HHV-7) from a healthy adult. Although the clinical symptoms caused by HHV-7 are not completely defined, we recently reported that HHV-7 also causes ES [Tanaka et al., 1994]. These two herpesviruses have similar characteristics such as T cell tropism, but they can be distinguished clearly by immunological and molecular biological techniques [Frenkel et al., 1990; Berneman et al., 1992]. Seroepidemiological investigations show that most children have antibodies against HHV-6 and HHV-7 by 2–3 years of age [Okuno et al., 1989; Briggs et al., 1990; Wyatt et al., 1991; Clark et al., 1993; Yoshikawa et al., 1993]. Saliva is the speculative source of transmission of both viruses because HHV-6 is detectable in saliva by polymerase chain reaction (PCR) [Jarrett et al., 1990; Gopal et al., 1990; Kido et al., 1990] and HHV-7 is isolated at the high frequency from the saliva of healthy adults [Wyatt and Frenkel, 1992; Black et al., 1993; Hidaka et al., 1993; Yoshikawa et al., 1993]. In this report, we describe the detection of HHV-6 and 7 in throat swabs of different aged children including ES patients, and in adults, using PCR. Since there is no seroepidemiological study in HHV-6 variants because of the difficulty of distinction of these vari-

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ants by the serological examination, this is the first report to suggest the prevalence of HHV-6 variant by PCR. We also discuss the pathogenesis of HHV-6 infection.

MATERIALS AND METHODS

Samples

Throat swabs were collected from a total of healthy children and 28 healthy adults. Of the 67 children, 32 were patients for the vaccination and/or the following of the development at our pediatric clinic, who ranged in age from 29 days old to 8 years old, and the remaining 35 were from an orphanage, and ranged in age from 1 month to 3 years of age. The two male and 26 female healthy adults were parents of our clinic patients. Children were classified into four groups by different ages: group I (15 children aged 29 days to 5 months); group II (15 children aged 6 months to 11 months); group III (23 children aged 1 year); group IV (14 children aged 2 years to 8 years); and adults (group V). Throat swabs were also collected serially from three patients with ES prior to and after the onset of the disease; these three patients were triplets who sequentially acquired ES. Peripheral blood was also collected from 54 children. Informed consents for blood and/or throat swab samplings were taken from all their mother and/or father or nurses.

Antibody Detection

The indirect immunofluorescence test for serum antibody to HHV-6 and 7 was performed as described previously [Asada et al., 1989; Tanaka et al., 1994]. Briefly, MT-4 cells, which were derived from an adult T-cell leukemia cell line, were infected with HHV-6, HST strain [Yamanishi et al., 1988], and Sup T1 cells, an immature T-cell line [Berneman et al., 1992], were infected with HHV-7, 7-KHR strain [Tanaka et al., 1994]. When cells showed cytopathic effect (CPE) with characteristic balloon-like syncytia, were mounted on 24-spot glass slides and fixed in acetone at −20°C for 15 min. Serially diluted sera was placed on the spotted slides and incubated for 1 hr at 37°C. The slides were then washed twice with phosphate-buffered saline (PBS), treated with antihuman IgG labeled with fluorescein isothiocyanate (Dako, Copenhagen, Denmark) and incubated for an additional 1 hr. After washing twice with PBS, the slides were observed under a fluorescent microscope. The results were read without knowing the age of the patient or their DNA shedding status to avoid bias.

Specimen Collection From Throat Swab

Throat swabs were collected with a sterile cotton stick by rubbing the throat. The cells on the cotton stick were suspended in 1.5 ml of sterile saline. Then the suspension was centrifuged at 10,000 rpm for 10 min at 4°C, and the pellets were resuspended with 100 μl of PBS. Ten μl of suspension was incubated for 10 min at 95°C, being put onto ice immediately and used in the PCR reaction.

Oligomer Synthesis

HHV-6 can be classified into HHV-6A and B using PCR primers described previously [Yalcin et al., 1994; Yamamoto et al., 1994]. The sizes of the first PCR amplification products of HHV-6A and -B were 325bp and 553bp and that of the nested double PCR amplification products of them were 195bp and 421bp, respectively. A part of HHV-7 major capsid protein gene was cloned and sequenced [Mukai et al., 1995]. The primers for first PCR were 5′-CACAAAAGCGTCGCTATCAA-3′(R3) and 5′-AGTTCCAGCACTGCAATCG-3′(F3). Those for nested double PCR were 5′-GACTCATTATGGGGATCGAC-3′(R4) and 5′-CGCATACACCAACCCTACTG-3′(F4). The size of the first PCR amplification products was 408bp, and that of nested double PCR amplification products was 264bp. The oligomers were synthesized on a DNA synthesizer (Applied Biosystems).

PCR

The PCR for HHV-6 was described previously [Kondo et al., 1990] and was used with some modifications as follows. The DNA thermal cycler conditions consisted of a 1-min denaturation step at 90°C, a 2-min annealing step at 62°C, and an elongation step of 3, 4, or 5 min at 72°C for 10 cycles. The DNA thermal cycler conditions for HHV-7 were a 1-min denaturation step at 94°C, a 1-min annealing step at 60°C, and a 1-min elongation step at 72°C for 30 cycles. The amplification reaction was carried using a thermal cycler (Hybaid, UK). The detection of DNA by PCR was attempted at least twice for each sample, and the results were the same on each occasion. Amplified samples were visualized on a 2% agarose gel electrophoresis with ethidium bromide staining.

Detection of Amplified Product

To confirm the results of PCR amplification, hybridization technique with alkaline phosphatase conjugated oligonucleotide probes was performed. The sequences of specific oligodeoxy-nucleotide probes for HHV-6 A and B and HHV-7 were 5′-TAAATCCATTACTGGCCTTGAA-3′ (HHV-6A), 5′-AACTCCATCAGCGGCCTCCAG-3′ (HHV-6B), and 5′-CATTACTCCAGTGACTTCCGATATTAATTT-3′, (HHV-7), respectively. Ten μl of PCR products were applied for electrophoresis, and DNA was transferred to a nylon membrane (Hybond N^+, Amersham). The membrane was treated with 0.4 N NaOH for 4 hr and then neutralized with 4xSSC (0.6 M NaCl, 0.06 M sodium citrate) for 15 min. After drying the membrane at 37°C for a few hours, prehybridization (5xSSC, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin fraction-V, and 1% sodium dodecyl sulfate (SDS)) was performed for 15 min at 50°C with shaking. Hybridization was performed with the equivalent of 100 ng/ml HHV-6A- and B- specific probes in sealed plastic bags containing with 5×SSC, 0.5% bovine serum albumine fraction-V, 0.5% polyvinylpyrrolidone, 1% SDS, 10 mM $MgCl_2$, 1 mM Zn

TABLE I. Prevalence of Antibody to HHV-6 and HHV-7 and Detection of DNA of Both Viruses in Throat Swabs by Age

Group	Age[a]	No. of subjects	DNA positive rate of HHV-6	DNA positive rate of HHV-7	Antibody positive rate of HHV-6	Antibody positive rate of HHV-7
I	0–5 M	15	2/15 (13.3)	0/15 (0)	8/12 (66.7)	6/12 (50.0)
II	6–11 M	15	10/15 (66.7)	2/15 (13.3)	10/12 (83.3)	4/12 (33.3)
III	12–23 M	23	20/23 (87.0)	13/23 (56.5)	15/16 (93.8)	12/16 (75.0)
IV	2–8 Y	14	11/14 (78.6)	11/14 (78.6)	13/14 (92.9)	13/14 (92.9)
V	adult	28	9/28 (32.1)	25/28 (89.3)	N.D.	N.D.

[a]M = month, Y = year.
N.D. = not done.

Cl_2, and 0.1% sodium azide for 15 min at 50°C with shaking. The membrane was washed and agitated once with 2×SSC plus 1% SDS for 10 min at 50°C once with 1×SSC plus 0.5% Triton X-100 for 10 min at room temperature, and placed into the color-producing reagents (0.3 M Tris-HCl[pH 9.8], 0.1 M NaCl, and 50 mM $MgCl_2$ with 0.33 mg/ml of nitro blue tetrazolium [NBT] and 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate [BCIP] [Sigma Chemical Co., St. Louis, MO]) for 30 min at 37°C without light. The reaction was terminated by washing in water for 15 min.

Specificity and Sensitivity of HHV-6 and HHV-7 Probes

The specificity of the PCR and the primers for HHV-7 were evaluated by hybridization against DNAs extracted from cells infected with human herpesvirus: herpes simplex virus types 1 and 2, varicella-zoster virus, human cytomegalovirus, Epstein-Barr virus, and HHV-6A and 6B; amplification was not detected even by Southern blot hybridization (data not shown). For determination of the sensitivity of the PCR in this system, samples of cloned HHV-6 and HHV-7 DNA were amplified with the primers of both viruses. HHV-6 DNA was detected at a level of ~10 copies after the nested double PCR, but the sensitivity of the PCR for HHV-7 was 10 times less (~100 copies) than for HHV-6 (data not shown).

Isolation of HHV-6

Peripheral blood was collected from triplets with ES, during the acute phase of the disease and the mononuclear cells were separated for virus isolation as described previously [Yamanishi et al., 1988].

DNA Analysis

The methods used for DNA purification of HHV-6 from infected cells, restriction-endonuclease digestion, and electrophoresis in an agarose gel have been described previously [Okuno et al., 1991; Mukai et al., 1994; Tanaka et al., 1994]. Briefly, umbilical cord blood mononuclear cells infected with HHV-6, ISS, ISM, and ISH strains, which were isolated from triplets with ES were solubilized in lysis buffer with deoxyribonuclease (DNase I: Boehringer Mannheim, Mannheim, Germany) and ribonuclease (RNase A; Sigma). Viral DNA was extracted with phenol, precipitated with ethanol, and centrifuged for pelleting. Restriction endonucleases, *Pst* I, *Bam* HI, and *Hind* III (Takara Chemical Co., Kyoto, Japan), were used for DNA digestion, and digested samples were loaded onto 0.6% agarose gels. Electrophoresis was conducted overnight at 4°C. The DNA fragments stained with ethidium bromide were visualized with an ultraviolet transilluminator and photographed.

RESULTS

Detection of HHV-6 and 7 DNA From Throat Swabs

HHV-6 DNA was detectable in 2/15 (13.3%) children in group I, 10/15 (66.7%) in group II, 20/23 (87.0%) in group III, and 11/14 (78.6%) in group IV, whereas DNAs were detected in 9/28 (32.1%) in healthy adults (group V). Among the children of group III, two children secreted HHV-6A and one child secreted HHV-6 both A and B. Two (group III) children from whom HHV-6A was detected and one (group IV) child from whom HHV-6A and B were detected had no history of ES. Two adults secreted HHV-6 both A and B DNA. The detection rate of HHV-7 DNA from throat swabs in groups I–V was 0/15 (0.0%), 2/15 (13.3%), 13/23 (56.5%), 11/14 (78.6%), and 25/28 (89.3%), respectively (Table I, Fig. 1).

Detection of Antibodies to HHV-6 and 7

The antibody titers of children were determined by IFA. For groups I–V, HHV-6 antibody was detected in 8/12 (66.7%), 10/12 (83.3%), 15/16 (93.8%), and 13/14 (92.9%), respectively, whereas HHV-7 antibody was detected in 6/12 (50.0%), 4/12 (33.3%), 12/16 (75.0%), and 13/14 (92.9%), respectively (Table I). Antibodies to HHV-6 and 7 in children, ages 0–5 months old, were considered to be maternal antibodies, but in the two cases of HHV-6 in which both the antibody titer and DNA shedding from throat swab were positive results, these may have been due to infection in the child (Table I).

Among triplets when the first patient had ES, blood samples were collected from the other siblings and their mother. The other two siblings and their mother's antibody titers to HHV-6 were <10 and 160, respectively. The antibody titers to HHV-6 among triplets rose from

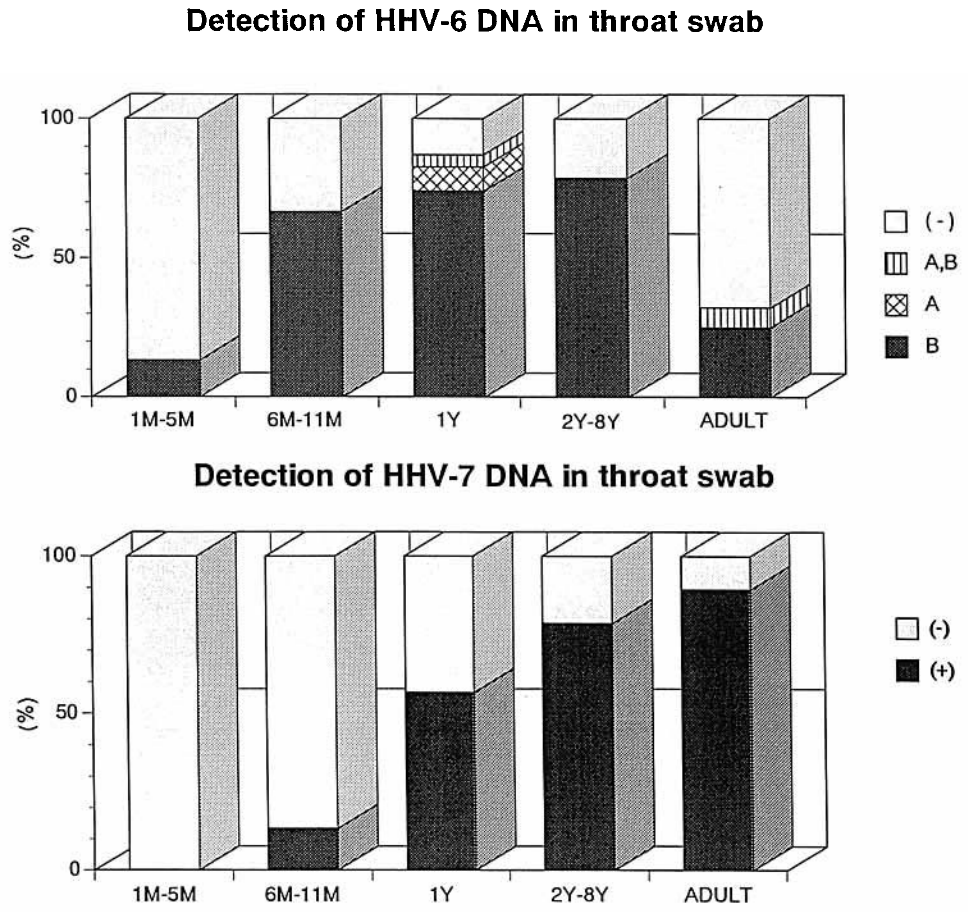

Fig. 1. Detection of HHV-6 and HHV-7 DNAs in throat swabs of children and adults by PCR. **A:** HHV-6A, **B:** HHV-6B, (−): not detectable, (+): detectable

<10 to >1,280 between the acute phase and the convalescent phase of ES (Fig. 2).

Detection of HHV-6 DNA in Samples of ES Patients

Samples were collected for PCR from triplets, from the first patient after the onset of disease, and second and third patient before and after the onset the disease. For all three patients, HHV-6 DNA was detected in all samples collected after the onset of the disease, although the copy numbers were highest during the acute phase. In contrast, no DNA was detectable in samples collected before the onset of the disease (Fig. 2). HHV-6 strains ISS, ISM, and ISH were isolated from all three patients during their acute phase of ES and during the convalescent phase the patients showed evidence of seroconversion to HHV-6. Three strains isolated from triplets had the identical patterns of the digestion by the restriction endonucleases of *Pst* I, *Bam* HI, and *Hind* III (Fig. 3).

DISCUSSION

Our seroepidemiological study showed that HHV-7 infection seems to occur later in life than HHV-6 infection, as reported by another group [Wyatt et al., 1991]. A recent study in the U.K., however, indicated no difference in the prevalence of antibody to both viruses by age [Clark et al., 1993]. No antibody was detectable before 2 yr old in the United States [Wyatt et al., 1991], but the percentage of HHV-7 positive children aged 12–23 months in Japan was 75% in our study (Table I), which is comparable to the finding by Yoshikawa et al. [1993]. However, the acquisition of antibody to HHV-7 seems to be earlier in our study. Methods of sample collection and a still limited amount of data may help to explain these discrepancies.

The mode of transmission of HHV-6 and 7 is not yet fully clear. We recently reported that the mode of transmission of HHV-6 appears to be horizontal, mainly from mother to child during infancy [Mukai et al., 1994], and sometimes from other babies in closed institutions such as orphanages, because we found an outbreak of ES in an orphanage and the DNA patterns of isolated viruses showed identical restriction enzyme patterns, suggesting the same virus was transmitted to other children [Okuno et al., 1991]. HHV-7 has been isolated frequently from the saliva of healthy adults [Wyatt and Frenkel, 1992; Black et al., 1993; Hidaka et al., 1993]. On the contrary, we found it extremely difficult to isolate HHV-6 [Mukai et al., 1994], although

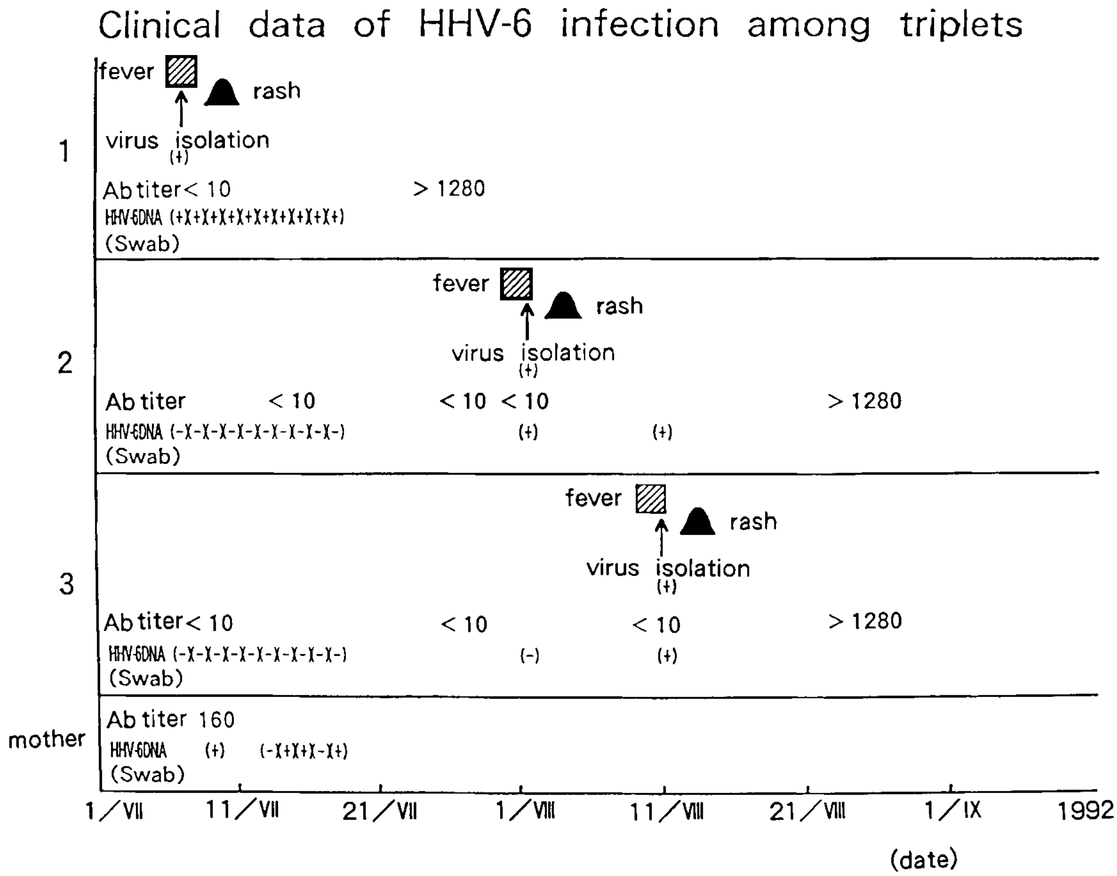

Fig. 2. Clincial course, antibody titer to HHV-6, virus isolation and detection of HHV-6 DNA in throat swabs from triplets and their mother.

several reports described the high rate of isolation of HHV-6 [Pietroboni et al., 1988; Harnett et al., 1990; Levy et al., 1990]. In our present report, the detection rate of HHV-7 DNA from throat swabs increased with age and reached its highest level in adults (89.3%). In contrast, the detection rate of HHV-6 was the highest in 1-year-olds (66.7%) and decreased in adults (32.0%). This result seems especially true, because the sensitivity of our PCR for HHV-6 was 10 times higher than for HHV-7 in our assay system as described in Materials and Methods. However, it is not clear why HHV-7 appears to infect later in life than HHV-6.

In this study, we found a correlation between HHV-6 variants and past history of ES and also reported the first known case of HHV-6A in Japan. The two children in group III who secreted HHV-6A had no history of ES, whereas the HHV-6 DNA detected from children who had a history of ES was all HHV-6B. Therefore, the clinical features of the primary HHV-6A infection are still not clear. One of the two children with HHV-6A DNA had a clinical history of Kawasaki-like disease, and the other one had only fever and common cold-like illness before the detection of HHV-6A DNA. Furthermore, based on these two samples, it seemed that HHV-6A infects later than HHV-6B, although the number of samples were not so many. It might be possible that the prevalence of HHV-6A in Japan is lower than that of HHV-6B, because the antibody to HHV-6B may protect against later infection by HHV-6A.

Concerning the site of the primary replication of HHV-6, HHV-6 DNA was not detectable in throat swabs of the second and third triplet patients from whom the samples were sequentially collected before onset of the disease. In varicella-zoster virus (VZV) infection, the primary site of replication is presumed to be the oropharyngeal area, because Ozaki et al. [1994] reported that viral DNA was detectable in throat swabs of samples collected even before the onset of varicella. On the contrary, HHV-6 was only detected after the onset of ES in our study, suggesting that HHV-6 may not replicate well in oropharyngeal area like VZV and it may be secreted from saliva after viremia during ES.

In conclusion, the detection rate of HHV-6 DNA was the highest in 1-year-olds, whereas that of HHV-7 increased with age, being highest in adults, and based on our seroepidemiological study, HHV-7 infection seemed to occur later in life than HHV-6 infection. We confirmed that a past history of ES was highly associated with the prevalence of HHV-6B infection. The highest copy numbers were detected during the acute and convalescent phases of the disease, but no DNA was detected in samples collected before onset of the disease.

Fig. 3. *Pst* I, *Bam* HI, an *Hind* III-cleaved DNA patterns from three isolates from triplets. MW: molecular marker, HST: a HHV-6 strain isolated from an ES patient, 1,2,3: triplets

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